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ORIGINAL PAPER

Polystyrene beads as an alternative support material for epitope identification of a prion-antibody interaction using proteolytic excision–mass spectrometry

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Abstract The binding epitope structure of a protein specifically recognized by an antibody provides key information to prevent and treat diseases with therapeutic antibodies and to develop antibody-based diagnostics. Epitope structures of antigens can be effectively identified by the proteolytic epitope excision–mass spectrometry (MS) method, which involves (1) immobilization of monoclonal or polyclonal antibodies, e.g., on *N*-hydroxysuccinimide-activated sepharose, (2) affinity binding of the antigen followed by limited proteolytic digestion of the immobilized immune complex, and (3) elution and mass spectrometric analysis of the remaining affinity-bound peptide(s). In the epitope analysis of recombinant cellular bovine prion protein ($bPrP^C$) to a monoclonal antibody (mAb3E7), we found that epitope excision experiments resulted in extensive nonspecific binding of bPrP to a standard sepharose matrix employed. Here, we show that the use of amino-modified polystyrene beads with aldehyde functionality is an efficient alternative support for antibody immobilization, suitable for epitope excision–MS, with complete suppression of nonspecific bPrP binding.

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Introduction

Aggregated amyloid-like proteins are involved in neurodegenerative diseases such as Parkinson's, Alzheimer's, Creutzfeldt-Jacob, and prion disorders. These diseases are believed to be caused by structural and/or conformational changes in proteins, leading to protein aggregation and finally to amyloid-like fibril formation [1, 2]. Prion diseases belong to the transmissible spongiform encephalopathies disease class and have been found both in humans and animals [3]. Fundamental insights into the mechanisms of these diseases are of paramount importance for the development of both diagnostic and effective therapeutic approaches.

Structural information on antigen–antibody interactions, particularly on antigen epitopes, is essential for the development of new diagnostics and for molecular vaccine design. A number of methods have been developed and are currently employed for the determination of antigen epitopes [4]. For example, a widely used epitope mapping technique is based on competition between overlapping synthetic peptides or subunits of recombinant peptide fragments and the complete antigen sequence for the antibody binding site. Major limitations of such techniques are that (1) they are only suitable for determination of linear epitope sequences, (2) partial sequences may fail to represent a complete recognition sequence, and (3) they are not suitable for identification of discontinuous epitopes, a type to which many epitopes belong [5]. High-resolution structural information of interacting residues of an antigen and an antibody can be obtained by X-ray crystallography and nuclear magnetic

resonance (NMR) spectroscopy [6]. However, these methods require high amounts of sample and a long time for analysis [7, 8]. Moreover, many protein complexes cannot be crystallized for structure determination, and NMR methods are strongly limited by available sample amounts, solubility issues, and the size of the proteins, which is particularly restrictive in the case of antigen–antibody complexes.

Epitope mapping approaches based on mass spectrometry have several advantages due to the molecular information obtained, high mass accuracy, short analysis time, sensitivity, and low sample consumption for the determination of linear, as well as discontinuous epitopes [6]. The proteolytic epitope excision–mass spectrometry approach [9] has been developed as a powerful method to directly identify amino acid sequences on an antigen involved in antigen–antibody interaction; this method involves limited proteolysis of the immune complex formed [10]. Antibodies are generally highly resistant towards proteolytic digestion and at the same time they protect the binding region on an antigen from possible cleavage by shielding. This approach has been successfully applied for epitope determinations on many peptide and protein antigens recognized by monoclonal as well as polyclonal antibodies [11–18]. Also, an antibody paratope has recently been identified using this technique [19]. However, when we applied this method for epitope excision on bovine prion protein (bPrP), abundant unspecific binding of bPrP to the sepharose material, most commonly used for antibody immobilization, was observed. Sepharose is a porous support material widely used for affinity chromatography that can "bury" protein molecules and therefore may lead to unspecific binding, in addition to specific binding to the antibody. The unspecific binding may be pronounced in the case of hydrophobic amyloid-like structures such as bPrP, which is known to readily aggregate.

Here, we present a high-efficiency epitope excision method for bPrP, by immobilizing the antibody on amino-modified polystyrene (PS) beads. The PS particles are frequently used as carriers of biomolecules in biomedical applications, mainly in immunoassays [20–22]. This approach showed a significant improvement of the epitope peptide identification from bPrP(25-241), recognized by the monoclonal antibody 3E7. Furthermore, the washing and elution steps were automated by centrifuging the beads using centrifugal filter units.

Experimental

Materials

Recombinant cellular bovine prion protein ($bPrP^C$) buffered in 10 mM sodium acetate at pH 4.0 and monoclonal anti-bPrP antibody mAb3E7 buffered in phosphate saline buffer (PBS) at pH 7.4 were obtained from Roboscreen (Leipzig, Germany). *N*-hydroxysuccinimide (NHS)-activated sepharose 4B was purchased from Sigma-Aldrich Chemie GmbH (Buchs SG, Switzerland). Amino-modified PS beads with a diameter of 0.65 μm were obtained from Bangs Laboratories Inc. (Fishers, IN, USA). "Mobicol" columns for affinity chromatography with a volume of 1 ml were obtained from MoBiTec GmbH (Göttingen, Germany). Centrifugal filter units with a molecular weight cutoff (MWCO) of 50 kDa "Microcon YM-50" and ZipTip C_{18} desalting pipette tips were obtained from Millipore (Billerica, MA, USA). Sequencing-grade chymotrypsin was purchased from Roche Diagnostics GmbH (Penzberg, Germany). C_{18} material for nano-LC-columns, Magic 3 μm 200 Å C18 AQ, was obtained from Bischoff GmbH (Leonberg, Germany).

Antibody immobilization on sepharose

Dry NHS-activated sepharose, 60 mg, was vigorously mixed with 200 μl of coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl in ultrapure H_2O at pH 8.3) containing 100 μg of mAb3E7 in a reaction tube for 2 h at room temperature. Covalent attachment of antibodies to the sepharose was achieved through primary amino groups of their N termini and lysine residues. The reaction mixture, mAb3E7 coupled to sepharose, was loaded into a "Mobicol" microcolumn. Unbound mAb3E7 was removed, and the remaining free primary amine groups on the sepharose were deactivated by subsequent washing of the column with 12 ml of blocking buffer (0.1 M ethanolamine, 0.5 M NaCl in ultrapure H_2O at pH 8.3) and 12 ml of washing buffer (0.2 M CH_3CO_2Na, 0.5 M NaCl in ultrapure H_2O at pH 4.0) using a syringe. The blocking–washing procedure was repeated seven times, and after the fourth time, the column was incubated for 1 h in blocking buffer followed by the washing step. Afterwards, the column was washed two times with 12 ml of PBS buffer (5 mM Na_2HPO_4, 150 mM NaCl in ultra pure H_2O at pH 7.5) and stored at 4 °C until further use. In the control experiments without mAb3E7, the procedure was exactly the same but no antibody was added to the coupling buffer.

Analysis of bPrP/mAb3E7 affinity

Following the mAb3E7 immobilization on sepharose, 10 μg of rec-$bPrP^C$ (5 μl of a 2 μg/μl protein solution) was diluted with 50 μl of PBS buffer at pH 7.5 in order to increase the pH of the protein solution and then added to the column. The mixture was incubated at room temperature for 2 h in PBS buffer with gentle shaking. The fraction of unbound bPrP was collected for further analysis by

sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Unbound bPrP was washed five times with 12 ml of PBS buffer and one time with ultrapure water. In order to control the efficiency of the washing step, the final aliquots of about 1 ml after both washings were collected. The bPrP-bound specifically to the mAb3E7 was eluted two times from the affinity medium with 500 μl of 0.1% trifluoroacetic acid (TFA) while gently shaking for 15 min. The collected fractions were lyophilized and then dissolved in 30–50 μl of loading buffer for SDS-PAGE analysis, which was carried out on a 12% gel according to Laemmli's method [23]. Protein samples were loaded on the gel at the volume of 15–20 μl. For molecular weight calibration, a commercial protein mixture in the range of 15–150 kDa was used. Proteins were visualized by silver staining [24].

Antibody immobilization on polystyrene beads

Immobilization of mAb3E7 on NH_2-activated PS beads and epitope excision were carried out in a 50-kDa MWCO Microcon centrifugal filter unit (0.5 ml). A "Mobicol" column was placed on the top of a filter in order to increase the volume (1 ml). The buffers used had the same composition as in the experiments on the sepharose column. First, 60 mg of beads was washed two times with 600 μl of washing buffer. Then, the beads were incubated with 10% solution of glutaraldehyde in washing buffer while gently mixing for 2 h at room temperature according to Bangs Lab's user guide in order to introduce a chemical spacer between the matrix and the antibody to avoid steric hindrance and therefore to maximize antibody binding. The beads were washed three times with 600 μl of washing buffer and three times with 600 μl of coupling buffer. All washing steps were conducted as follows: 600 μl of buffer was added to the beads in the Microcon centrifugal filter unit, mixed for 2 min and centrifuged at 7,500 rpm for about 5 min until the initial volume was re-established. Antibody, 100 μg of mAb3E7 in coupling buffer, was added as described for antibody immobilization on sepharose. Blocking of free amine groups and washing steps were carried out in the similar way as described for the sepharose experiment with difference in the applied volumes and use of centrifugation.

Epitope excision

Affinity binding of rec-bPrP^C to the immobilized mAb3E7 on sepharose or on PS beads was carried out in a similar way: 10 μg of rec-bPrP^C in 50 μl of PBS buffer was added to the mAb3E7-immobilized column in the case of sepharose or to the mAb3E7-immobilized centrifugal filter unit in the case of PS beads. The mixture was incubated at room temperature for 2 h with gentle shaking. Afterwards, unbound bPrP was removed by washing five times with 12 ml of PBS buffer in the case of a sepharose column and ten times with 600 μl of PBS buffer in the case of a beads column.

Sequencing-grade chymotrypsin dissolved in PBS buffer was added to the affinity-bound bPrP/mAb3E7 complex formed to give an enzyme to bPrP protein ratio of 1:20 (*w*/*w*). Proteolysis was carried out at 25 °C overnight. After digestion, the sepharose column was washed ten times with 12 ml of PBS buffer and three times with 12 ml of ultrapure water to remove the proteolytic products. The centrifugal filter unit with beads was washed five times with 600 μl of PBS buffer and five times with 600 μl of ultrapure water. The first 1 ml of the digest (supernatant) and the last 1 ml of the washing fraction with water ("wash") were collected in order to control both the digestion and washing steps. The bPrP fragments that were protected from chymotryptic proteolysis by mAb3E7 were then eluted two times with 500 μl of 0.1% TFA ("elution") while gently shaking for 15 min. The collected fractions were lyophilized and later dissolved in 10 μl of 0.1% TFA for further analysis by nano liquid chromatography–electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (nano-LC–ESI–FTICR MS).

Nano-LC–ESI–FTICR mass spectrometry

The peptides generated by proteolytic digestion were separated in a nano high-performance liquid chromatography (nano-HPLC) system (Eksigent Technologies, Dublin, CA, USA) using self-made reversed-phase columns packed with C18 material. ESI–FTICR MS analysis was carried out on a commercial FTICR mass spectrometer (LTQ-FTMS, Thermo Finnigan, Bremen, Germany) coupled to the nano-HPLC. This technique is very sensitive, allowing attomole detection and providing high resolution and mass accuracy necessary for unambiguous identification of the resulting peptides. Before analysis, every sample was cleaned up with ZipTip C18 pipette tips (Millipore) according to the manual of the manufacturer. Ions in the mass-to-charge range (*m*/*z*) from 300 to 2,000 Da were acquired, and MS/MS data were collected using collision-induced dissociation (CID). Low-energy CID of the peptides was conducted in the ion trap. Conditions for MS/MS acquisitions were as follows: normalized collision energy, 35%; ion selection threshold, 500 counts; activation q=0.25; and activation time 30 ms. Target ions already selected for MS/MS were dynamically excluded for 60 s.

Peptide assignment was achieved using the MS/MS ion search option of MASCOT (Matrix Science Inc., Boston, MA, USA) search engine and the SwissProt database. A mass tolerance of ±5 ppm for peptide

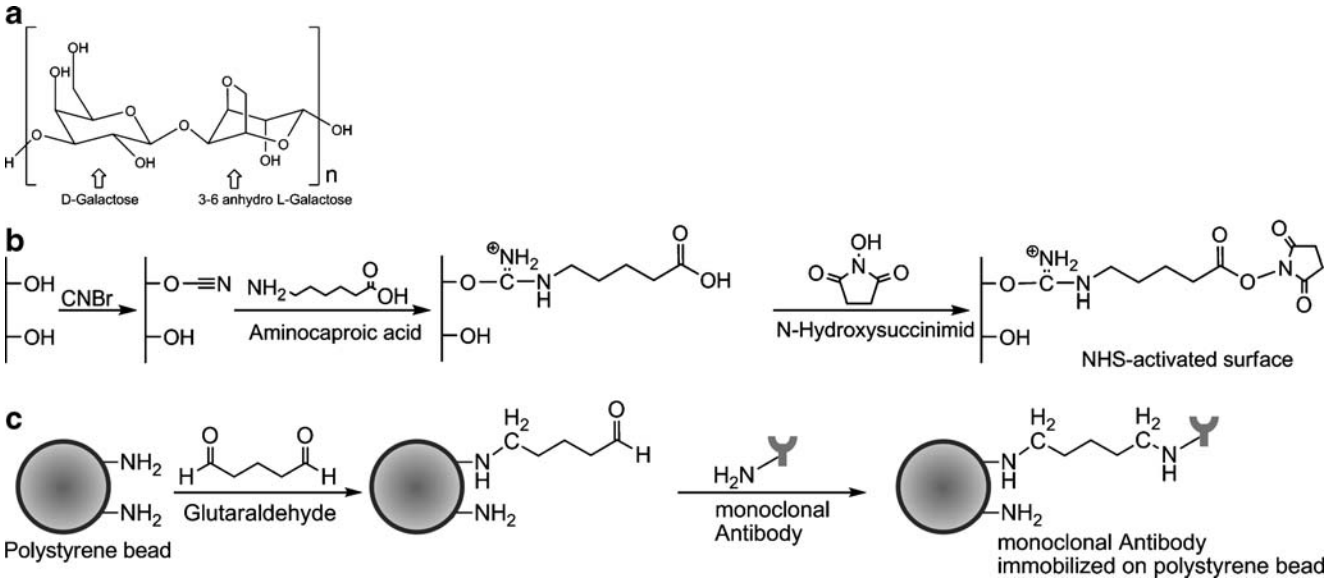

Fig. 1 **a** Chemical structure of sepharose and **b** scheme of derivatization of *N*-hydroxy-succinimide (NHS)-activated surface on sepharose. **c** Schematic representation of antibody immobilization on NH_2-activated PS beads using glutaraldehyde as a spacer

precursor ions and of ±0.6 Da for MS/MS fragments was used for peptide identification, allowing up to three missed cleavages. The absolute peak intensities averaged over the elution time of each of the identified bPrP peptides were extracted manually from each LC–MS run using the "extracted ion chromatogram" option of XCalibur 2.0 (Thermo Finnigan, Bremen, Germany), the instrument-specific software. Peak intensities below 500 a.u. were considered as a noise.

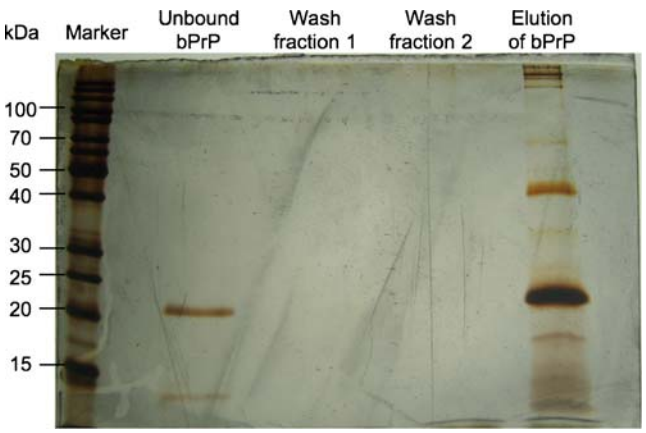

Fig. 2 Silver-stained 12% SDS-PAGE demonstrates the affinity between the immobilized mAb3E7 and the bPrP(25-241). Molecular weight markers (in kilodalton) are shown on the left side of the gel, and each line is labeled according to the sample applied. The major gel band corresponds to bPrP, which was detected following elution by acidification of bPrP/mAb3E7 complex. Washing fractions before elution were also analyzed (*lanes 3* and *4*), and they show no unbound bPrP in the affinity medium

Results and discussion

The epitope excision–MS approach was applied to first verify the linear epitope on bPrP(25-241) recognized by mAb3E7, which has been determined earlier in our group as peptide [150–160] by an indirect approach combining chemical cross-linking with mass spectrometry [25]. The epitope excision method was chosen because "native-like" environment can be used to preserve the conformation of both proteins and thus allow the affinity interaction of an antigen with its antibody. Conditions for proteolytic digestion of bPrP(25-241) in the absence of the antibody have been previously optimized [25] and used in the following experimental procedures. Chymotrypsin was the enzyme of choice here, as it generates peptides in the appropriate size and mass range for identification by LC–ESI–FTICR MS, resulting in a higher sequence coverage of bPrP compared to tryptic digestion. The greater part of bPrP(25-241) was completely digested at the conditions employed. This was monitored by SDS-PAGE, by comparing gel bands which corresponded to aliquots of digested bPrP after 2, 4, 6 h and overnight with a gel band of undigested bPrP at about 24 kDa (data not shown). The conditions of chymotryptic digestion were deemed appropriate when no gel band was observed at 24 kDa and when resulting peptides were identified unambiguously by LC–ESI–FTICR MS.

In the epitope excision experiments, the antibody was first immobilized on NHS-activated sepharose, which is a macroporous chromatography matrix with a high chemical and physical stability. NHS activation of sepharose is

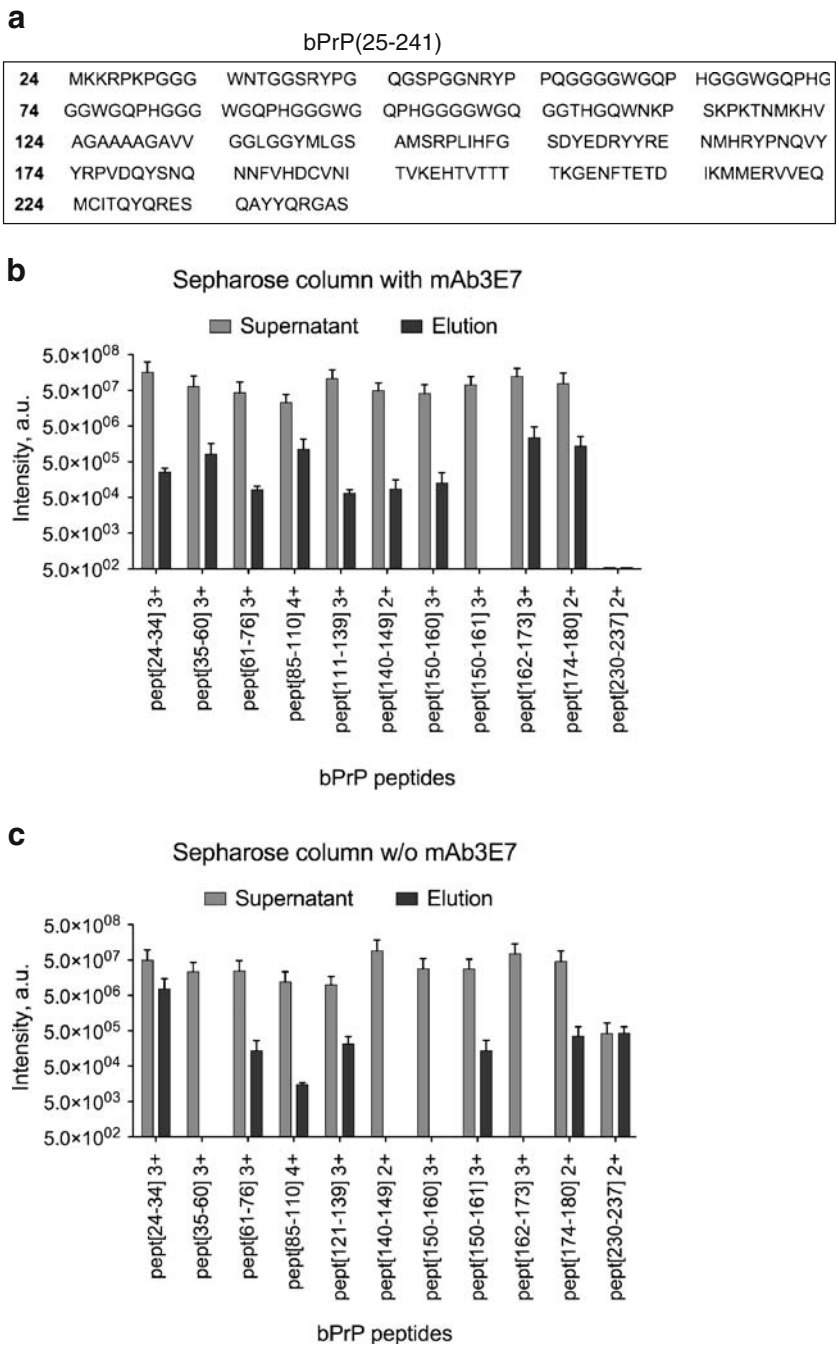

Fig. 3 **a** Amino acid sequence of bPrP(25-241). **b**, **c** Diagrams displaying the mean values with the standard error of the mean of the absolute peak intensities for each of the identified bPrP(25-241) peptide in the supernatant (*gray bars*) and in the elution fractions (*black bars*), shown on a logarithmic scale. The data were obtained from three independent experiments on chymotryptic epitope excision–mass spectrometry on a sepharose column **b** with immobilized mAb3E7 and **c** without the antibody immobilized. There were no bPrP peptides detected in the washing fraction before elution. Each peptide was identified at least in two experiments and depicted with its numbers assigned corresponding to the position in the bPrP sequence and with its charge state. Signal intensities below 500 arbitrary units were considered as noise

schematically shown in Fig. 1a, b where hydroxyl groups of the sugar residues are used for modification. The antigen, bPrP(25-241), whose amino acid sequence is given in Fig. 3a, was added to the microcolumn. The affinity between the immobilized mAb3E7 and the bPrP was confirmed by SDS-PAGE (Fig. 2). The most abundant gel band corresponds to bPrP, which was eluted from the antibody with 0.1% TFA, although it was not as intense as expected. A small part of unbound bPrP was left after incubation of mAb3E7 and bPrP, as displayed on the gel. This suggests that the amount of bPrP added to the mAb3E7-immobilized column was sufficient, and most probably both specific interaction of bPrP with the antibody and unspecific binding with sepharose took place.

Chymotryptic epitope excision was conducted as described in the experimental part. Following the collection of bPrP peptides after proteolysis (supernatant), the microcolumn was extensively washed with PBS buffer, and the last fractions were analyzed by LC–ESI–FTICR MS until

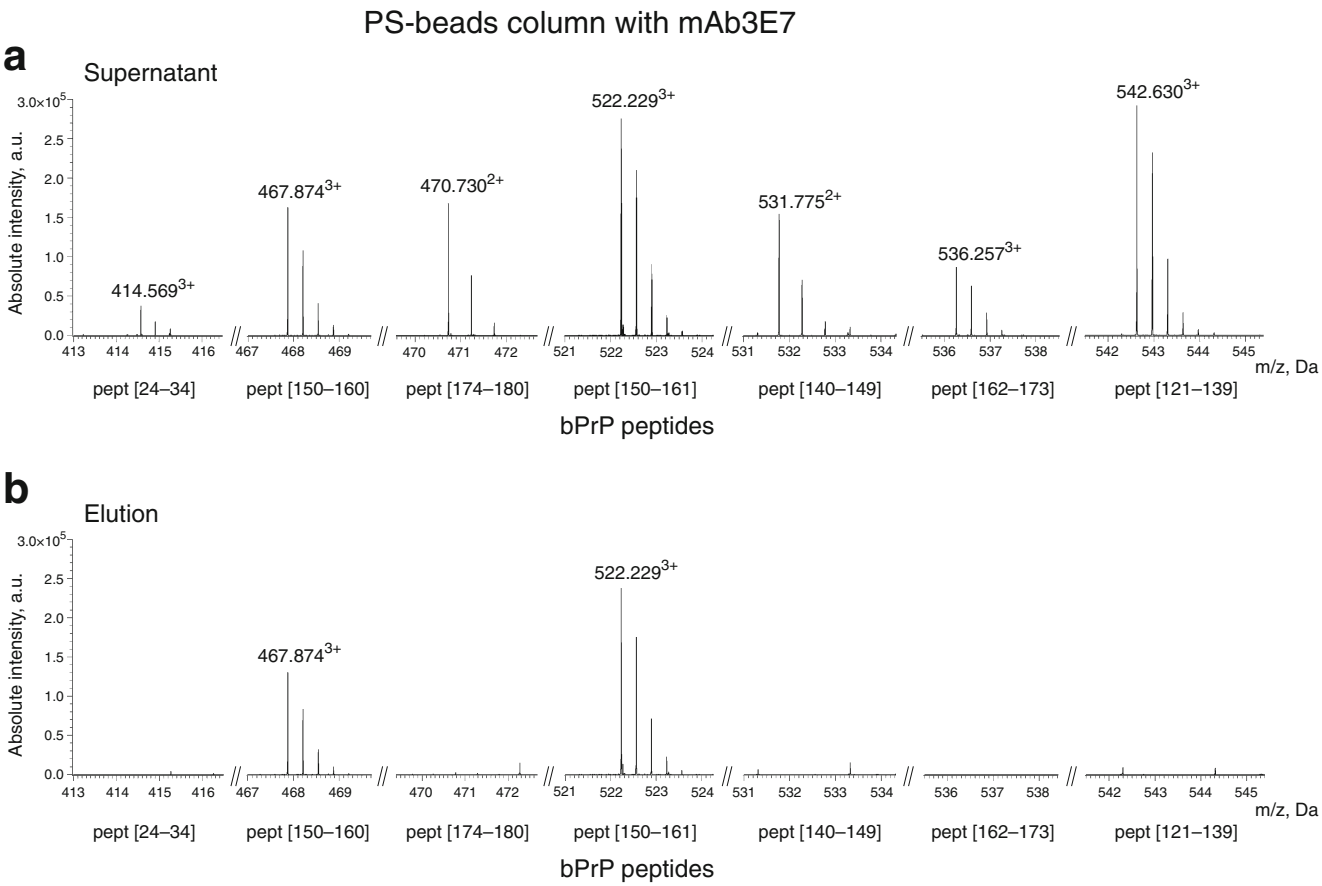

Fig. 4 Sections of LC–ESI–FTICR mass spectra derived from extracted ion chromatograms for each of identified bPrP(25-241) peptides with their *m/z* values and charge states **a** in the supernatant and **b** in the elution fractions, obtained from chymotryptic epitope excision on PS beads affinity column with immobilized mAb3E7. There were no bPrP peptides detected in the washing fraction before elution

bPrP peptides were no longer detected. The bPrP fragments constituting the epitope were eluted from the affinity medium by acidification. The data summarized in Fig. 3b display the mean values with the standard error of the mean of absolute peak intensities extracted from LC–MS runs of each of the identified bPrP peptides presented in the supernatant (gray bars) and in the elution (black bars) fractions from three independent experiments. The peptides identified are almost the same in both fractions and among them the known epitope peptide [150–160], although the elution fraction was expected to contain only epitope specific peptide(s). A strong reduction in the peak intensities of the eluting peptides was also observed, possibly due to dilution of the bPrP peptides by the extensive washing steps before elution. These results indicate significant nonspecific binding of bPrP to the sepharose matrix, suggesting that epitope identification would be difficult at these conditions.

To verify nonspecific binding, we conducted experiments following the same protocol but in the absence of antibody in the coupling buffer. We found the same bPrP peptides in the supernatant as in the experiments with mAb3E7, corresponding to 67% of the bPrP(25-241) sequence (Fig. 3c) and at the comparable peak intensities. This indicates that quite significant nonspecific binding with sepharose material occurred. While the open-pore structure of sepharose ensures high-capacity binding for large biomolecules such as antibodies, it may lead to unspecific binding, e.g., of the highly hydrophobic peptides produced by digestion of bPrP(25-241).

Antibodies can also be immobilized on PS beads, a procedure which is often used in biomedical diagnostics [20, 21]. Here, we introduce NH_2-activated PS beads as an alternative matrix for epitope excision. PS beads represent a nonporous immobilization support for antibodies and we reasoned that antigen molecules would stick less to the bead surface. In order to avoid “steric crowding” when coupling antibody molecules and to facilitate effective binding of an antigen to the antibody, a glutaraldehyde spacer was introduced between the beads matrix and an

antibody, as depicted in Fig. 1c. Covalent attachment of mAbs takes place through reaction between the surface aldehyde groups of the PS beads and the amino groups of lysines and N terminus.

To investigate nonspecific binding effects of bPrP(25-241) to the PS bead matrix modified with glutaraldehyde, a control proteolytic experiment without antibody mAb3E7 was conducted as described in the experimental part. No bPrP peptides were detected, neither in the supernatant nor in the elution fraction, demonstrating the absence of nonspecific adsorption. This is probably due to the much smaller specific surface of the beads. The efficiency of PS beads as a chromatography matrix for epitope excision of bPrP(25-241) was then tested with mAb3E7 immobilized, using the very similar experimental conditions as in the experiments with the sepharose matrix. A summary of the bPrP(25-241) peptides identified in the supernatant and in the elution fractions is shown in Figs. 4a, b, respectively. The absolute peak intensities averaged over the elution time of each of the bPrP(25-241) peptides are presented as partial ESI–FTICR mass spectra. Only two specific peptides [150–160] and [150–161] with overlapping sequences were detected in the elution fraction. They correspond to the mAb3E7 epitope which we have determined earlier using the indirect approach based on chemical cross-linking and mass spectrometry. A part of this sequence, bPrP peptide [150–155], was also deduced from the PEPSCAN method [26] as an epitope on bPrP recognized by the mAb3E7. Therefore, our data are in complete agreement with previously obtained results.

Conclusions

This study shows the advantages of using PS beads as an affinity chromatography matrix when determining an epitope on bPrP by limited proteolysis and mass spectrometric peptide mapping method (epitope excision). Nonspecific binding of bPrP molecules was not observed when employing this column material. The usage of PS beads is a better alternative to the sepharose matrix in the case of proteins with a strong tendency to aggregate like bPrP or to interact with carbohydrate matrices.

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